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<p>(54) Title: METHODS AND COMPOSITIONS FOR INDUCING ORAL TOLERANCE IN MAMMALS</p>		
<p>(57) Abstract</p> <p>The present invention relates to methods and pharmaceutical formulations for orally delivering an antigen to induce tolerance. The antigen is combined with derivatized amino acids or salts thereof. The induction of oral tolerance may be applied clinically for the prevention or treatment of auto-immune diseases and clinical allergic hypersensitivities, and for the prevention of allograft rejection. The figure illustrates examples of the derivatized amino acids.</p>		
<div style="display: flex; flex-wrap: wrap; justify-content: space-around;"> <div style="text-align: center; margin: 5px;"> <p>E352</p> </div> <div style="text-align: center; margin: 5px;"> <p>E597</p> </div> <div style="text-align: center; margin: 5px;"> <p>E445</p> </div> <div style="text-align: center; margin: 5px;"> <p>E579</p> </div> <div style="text-align: center; margin: 5px;"> <p>E94</p> </div> <div style="text-align: center; margin: 5px;"> <p>E198</p> </div> <div style="text-align: center; margin: 5px;"> <p>E594</p> </div> <div style="text-align: center; margin: 5px;"> <p>E702</p> </div> <div style="text-align: center; margin: 5px;"> <p>E623</p> </div> </div>		

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METHODS AND COMPOSITIONS FOR INDUCING ORAL TOLERANCE IN MAMMALS

FIELD OF THE INVENTION

5 The present invention relates to methods and compositions useful for the induction of oral tolerance to a coadministered antigen in mammals.

BACKGROUND OF THE INVENTION

 Immunological antibody responses to pathogens are required to prevent infections in the body, whereas, immunological tolerance is a property of the immune system
10 that allows for the discrimination of self from non-self. A breakdown in immunological tolerance to self antigens allows the onset of anti-self immunological responses through the generation of anti-self antibodies and/or cellular immune responses. This breakdown is responsible for auto-immune diseases seen in both humans and other mammals.

 Allergic immune responses to allergens such as those classically observed in,
15 for example, hay fever, reactions to insect bites and common food allergies is suppressed through the generation of immunological tolerance to the antigen responsible for the allergy, i.e., the allergen. Repeated exposure to a particular allergen through controlled administration of allergen can induce tolerance in some patients.

 Oral tolerance has been characterized in the literature as a state of antigen-
20 specific systemic immunological unresponsiveness or tolerance, which is induced by prior oral administration or feeding of antigen. (A.M. Mowat, *Immunology Today*, Vol. 8, No. 3, 1987, pp. 93-98.) Such a state of systemic hypo-responsiveness to an administered protein or antigen has been observed and reviewed in the art. (H.L. Weiner, *Proc. Natl. Acad. Sci. USA*, Vol. 91, 1994, pp. 10762-10765; and H.L. Weiner and L.F. Mayer, eds., *Annals of NY Acad. Sci.*, Vol. 778, 1996, pp. xiii-xviii.)
25

 It has been shown that oral co-administration of antigens with cholera toxin B subunit as an adjuvant provides an efficient transmucosal delivery system for induction of immunological tolerance. (J.B. Sun, et al., *Proc. Natl. Acad. Sci. USA*, Vol. 91, 1994, pp. 10795-10799; and C. Czerkinsky, et al., *Annals NY Acad. Sci.*, Vol. 778, 1996, pp. 185-
30 193.) In these studies sheep red blood cells (SRBC), horse red blood cells (HRBC) or purified human gamma-globulin (HGG) were used as antigen and covalently conjugated to the cholera toxin B (CTB) subunit delivery agent. The SRBC-CTB, HRBC-CTB or HGG-CTB were administered orally to mice to induce oral tolerance to these antigens.

 One example of a inflammatory demyelinating autoimmune disease in humans
35 is Multiple Sclerosis. Experimental Autoimmune (a.k.a. Allergic) Encephalomyelitis (EAE) is a paralytic disease of the central nervous system (CNS) that can be induced in animals by

injection, together with Complete Freund's Adjuvant, of brain or spinal cord homogenate, purified Myelin Basic Protein (MBP) or other purified encephalitogenic proteins (derived from brain or spinal cord) or synthetic peptides whose amino acid sequences resemble those of encephalitogenic components of CNS tissues. EAE is widely used as a model for human autoimmune inflammatory demyelinating disorders such as Multiple Sclerosis (J-B. Sun, et al, Proc. Nat'l Acad. Sci., USA, 93, 7196-7201 (1996)). Certain strains of animals display greater susceptibility to the disease, including Lewis rats and SJL/J mice. Cats, dogs, Guinea Pigs, rabbits and marmoset monkeys may also be susceptible. The most common source of active encephalitogens is Guinea Pig brain or spinal cord.

The encephalitogen/adjuvant suspension is injected into the footpads of experimental animals, inducing the onset of disease symptoms within 10-12 days. Prevention or modulation of EAE symptoms has been achieved by induction of oral tolerance via oral administration of large, numerous doses of MBP either before or after induction of the disease. Generally, at least five oral doses are required. Determination of synergistic or immune enhancing agents to be administered together with MBP in order to reduce the number or magnitude of the MBP doses required to modulate the disease symptoms is desirable. In addition, agents that can deliver consistent concentrations of antigen to the gut are also desirable. If such agents could be identified, immunogenic tolerance to these and other types of autoimmune diseases could be promoted.

SUMMARY OF THE INVENTION

The present invention relates to methods and formulations for inducing oral tolerance in an animal, comprising orally administering to the animal a pharmaceutical formulation comprising an antigen and a delivery agent or agents comprising at least one derivatized amino acid or a salt thereof in an amount sufficient to induce oral tolerance. These delivery agents allow the administration of lower or fewer doses of antigen than are required to induce the same degree of systemic immune suppression with the antigen alone. The immune responses involved include, but are not limited to, systemic antibody production or delayed-type hypersensitivity reactions. In addition, the antigens for use in the induction of oral tolerance do not have to be covalently linked to the delivery agents.

It is believed that the foregoing delivery agents, when used in the proportions noted below, enhance the action of the antigens by increasing the proportion of ingested antigen which reaches the systemic circulation in its tolerogenic form. It may be that this is achieved by stabilization by the delivery agent of the tolerogenic form or fraction of the antigen in a configuration which may more easily cross the mucosal epithelium. It will be understood that the methods and compositions of the invention are not limited by the foregoing possible mode of action.

The invention relates to methods of inducing oral tolerance in an animal wherein the derivatized amino acid is comprised of an amino acid bearing a free carboxyl group, an amide linkage and a hydrophobic chain comprised of aromatic and/or aliphatic components.

- 5 A preferred embodiment of the invention relates to methods of inducing oral tolerance in an animal wherein the derivatized amino acid is an acylated amino acid compound of the formula



wherein:

- 10 Ar is a substituted or unsubstituted phenyl,
 R is $\text{NH}(\text{R}^1)\text{-C-}$
 R^1 is C_1 to C_{10} alkyl, C_1 to C_{10} alkenyl, phenyl, naphthyl, (C_1 to C_{10} alkyl) phenyl, (C_1 to C_{10} alkenyl) phenyl, (C_1 to C_{10} alkyl) naphthyl, (C_1 to C_{10} alkenyl) naphthyl;
 R^1 is optionally substituted with C_1 to C_4 alkyl, C_1 to C_4 alkenyl, C_1 to C_4 alkoxy, -OH, -SH and $-\text{CO}_2\text{R}^2$, cycloalkyl, cycloalkenyl, heteroalkyl, alkaryl, heteroaryl,
 15 heteroalkaryl, or any combination thereof; and
 R^2 is hydrogen, C_1 to C_4 alkyl or C_1 to C_4 alkenyl.

- Another preferred embodiment of the invention relates to methods of inducing oral tolerance in an animal wherein the derivatized amino acid is a sulphonated amino acid
 20 compound of the formula



wherein Ar and R are as defined above.

Examples of the aforementioned derivatized amino acids are described in Figure 1 below and include:

- 25 4-[4-(N-salicyloyl)]aminophenyl butyric acid (E352);
 N-salicyloyl phenylalanine (E94);
 4-[4-(N-benzenesulfonyl)] aminophenyl butyric acid (E198);
 3-[4-(N-2,3-dimethoxybenzoyl)] aminophenyl propionic acid (E702);
 10-(N-salicyloyl) amino decanoic acid (E597);
 30 4-[4-(N-4 phenylbutyryl)] aminophenyl butyric acid (E445);
 4-[4-(N-2 methoxybenzoyl)] aminophenyl butyric acid (E579);
 3-[4-(N-2 methoxybenzoyl)] aminophenyl propionic acid (E594); and
 4-[4-(N-phenoxyacetyl)] aminophenyl butyric acid (E623).

- The present invention also relates to pharmaceutical formulations for inducing
 35 oral tolerance in an animal, comprising an antigen and a delivery agent or agents comprising

at least one derivatized amino acid or a salt thereof in an amount sufficient to induce oral tolerance. Preferably, the invention relates to pharmaceutical formulations for inducing oral tolerance, wherein the derivatized amino acid is administered at a dose of about 100-1000 mg per kg of the subject's body weight, preferably at a dose of about 250-750 mg per kg of body weight.

Also contemplated are methods and pharmaceutical preparations incorporating an adjuvant or adjuvants with the antigen and delivery agent or agents. The formulations are particularly advantageous for inducing oral tolerance to antigens which otherwise would require large and/or chronic dosing of antigen to induce such tolerance and which, by themselves, do not pass or are not taken up in the gastrointestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes in the gastrointestinal tract. Such antigens include those associated with or responsible for the induction of auto-immune diseases, clinical (allergic) hypersensitivities, and allograft rejection, and subunits or extracts therefrom; or recombinantly generated whole proteins, subunits or fragments thereof; or any combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The details of the present invention will be more fully described in connection with the accompanying drawings, in which:

Figure 1 provides formulas of preferred derivatized amino acids useful in the invention.

Figure 2 is a graphic representation of the titer of serum anti-sheep red blood cell (anti-SRBC) IgG antibodies (determined through the indirect IgG assay) and IgM antibodies (determined through the direct IgM assay) in mice fed SRBC with or without salicyloyl-phenylalanine (E94) derivatized amino acid delivery agent in accordance with Examples 1 and 2 and Comparative Examples A, B, C, and D.

Figure 3 is a graphic representation of the titer of anti-influenza antibodies (determined through the hemagglutination inhibition assay) present in pooled sera of mice pre-immunized with influenza antigen and fed influenza antigen with or without salicyloyl-phenylalanine (E94) derivatized amino acid delivery agent in accordance with Example 3 and Comparative Example E.

Figure 4 is a graphic representation of the titer of serum anti-HA (influenza) IgG antibodies following single-dose feeding of influenza vaccine A/Johannesburg/39/94 (H3N2) with or without N-salicyloyl-4-amino-phenyl butyric acid (E352) derivatized amino acid delivery agent in accordance with Example 4 and Comparative Example F.

Figure 5 is a graphic representation of the titer of serum anti-ovalbumin IgG antibodies at week 12 in mice fed two doses of ovalbumin at weeks 0 and 4 with or without

salicyloyl-phenylalanine (E94) derivatized amino acid delivery agent and challenged intramuscularly at week 9 with ovalbumin and Complete Freund's Adjuvant (CFA) in accordance with Example 5 and Comparative Examples G and I.

5 Figure 6 is a graphic representation of the titer of serum anti-ovalbumin IgG antibodies at week 4 in mice fed a single dose of ovalbumin with or without salicyloyl-phenylalanine (E94) derivatized amino acid delivery agent and challenged subcutaneously at week 1 with ovalbumin in CFA in accordance with Example 6 and Comparative Examples H and I.

10 Figure 7 is a graphic representation of the titer of serum anti-sheep red blood cell (anti-SRBC) IgG antibodies (determined through the indirect IgG assay) on day 14 in mice fed SRBC with or without 3-[4-(N-2 methoxybenzoyl)] aminophenyl propionic acid (E594) derivatized amino acid delivery agent in accordance with Example 7 and Comparative Examples J and K.

15 Figure 8 is a graphic representation of footpad thickness in a DTH assay at day 14 in mice fed a single dose of SRBC with or without 4-[4-(N-benzenesulfonyl)] aminophenyl butyric acid (E198) and challenged in the footpad at day 7 in accordance with Examples 8 and 9 and Comparative Examples L and M.

20 Figure 9 is a graphic representation of footpad thickness in a DTH assay at week 5 in mice fed a single dose of ovalbumin with or without 3-[4-(N-2,3-dimethoxybenzoyl)] aminophenyl propionic acid (E702) derivatized amino acid delivery agent and challenged subcutaneously at week 3 with ovalbumin in CFA in accordance with Example 10 and Comparative Examples N and O.

25 Figure 10 is a graphic representation of the Mean Clinical Score for the progression of EAE over time in Lewis rats fed a single dose of MPB with N-salicyloyl phenylalanine (E94) or 3-[4-(N-2,3-dimethoxybenzoyl)] aminophenyl propionic acid (E702) derivatized amino acid delivery agents; MBP alone in 1 dose; or MBP given in 5 doses in accordance with Example 11.

30 Figure 11 is a graphic representation of the Mean Clinical Score for the progression of EAE over time in Lewis rats fed a single dose of MPB with 4-[4-(N-salicyloyl)]aminophenyl butyric acid (E352) derivatized amino acid delivery agent; MBP alone in 1 dose; or MBP given in 5 doses in accordance with Example 11.

35 Figure 12 is a graphic representation of the mean Maximal Score per group and the Mean Disease Index per group (defined as the highest mean score multiplied by the duration of symptoms) for Lewis rats dosed with a single dose of MBP and with 4-[4-(N-salicyloyl)]aminophenyl butyric acid (E352) derivatized amino acid delivery agent; MBP alone in 1 dose; or MBP given in 5 doses in accordance with Example 11. The subscripts in

the group labels refer to the number of paralyzed rats in each group, and the "% protection", i.e. the percent of animals that were not paralyzed.

DETAILED DESCRIPTION OF THE INVENTION

The present invention uses readily available and inexpensive delivery agents to provide animals with oral tolerance to antigens. Oral tolerance is characterized as a state of antigen-specific systemic immunological hypo-responsiveness induced by the feeding of an antigen. Oral tolerance generally results from large or chronic doses of antigens. As pointed out hereinabove, the present invention is directed to methods and pharmaceutical formulations comprising an antigen and an derivatized amino acid or salt delivery agent useful to induce oral tolerance to the antigen when the antigen and delivery agent are fed simultaneously. The delivery agents allow the administration of lower or fewer doses of antigen than are required to induce the same degree of systemic immune suppression with the antigen alone. The delivery agents allow the administration of more consistent dosing concentration of antigen. The immune responses involved include, but are not limited to, systemic antibody production and delayed-type hypersensitivity reactions.

The induction of oral tolerance may be applied clinically for the prevention or treatment of auto-immune diseases and clinical (allergic) hypersensitivities, and for the prevention of allograft rejection.

Antigens

Antigens suitable for use in the present invention include, but are not limited to, synthetic or naturally derived proteins and peptides, and particularly those which by themselves require high doses to induce oral tolerance; carbohydrates including, but not limited to, polysaccharides; lipopolysaccharides; and antigens isolated from biological sources such as, for example, those associated with or responsible for the induction of auto-immune diseases, clinical (allergic) hypersensitivities, and allograft rejection and subunits or extracts therefrom; or any combination thereof.

Special mention is made of antigens associated with the autoimmune diseases of multiple sclerosis, lupus erythematosus, scleroderma, uveitis, insulin-dependent diabetes mellitus or arthritis. In addition, self-antigens include: nucleic acid; oligodeoxynucleotide; thyroglobulin; thyroid cell surface or cytoplasm; parietal cell; adrenal cell; epidermal cell; uvea cell; basement membrane cell; red cell surface; platelet cell surface; muscle cell; thymus myeloid cell; mitochondria; secretory duct cell; deoxyribonucleic acid-protein; acetylcholine receptor substance; insulin; central nervous system antigens such as, myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein; and other normal hormone and tissue factors.

Allergens include: benzylpenicilloyl, insulin, ovalbumin, lactalbumin, bermuda grass pollen, timothy grass pollen, orchard grass pollen, and combinations of grass pollen, ragweed pollen, ragweed antigen E, birch tree pollen, bee venom, snake venom, horse dander, cat epithelial, haddock, house dust mite, *Chrysanthemum leucanthemum*, *Alternari tenuis*, trypsin, chymotrypsin, dry rot, baker's yeast, tetanus toxoid, diphtheria toxin, ficin and derivatives thereof.

Delivery Agents

The delivery agents employed in the practice of the present invention are derivatized amino acids or salts thereof. The derivatized amino acids include amino acid amides.

Amino acids which may be used to prepare the delivery agents employed in the methods and compositions of the invention include any carboxylic acid having at least one free amino group, including both naturally occurring and synthetic amino acids. Many amino acids and amino acid esters are readily available from a number of commercial sources such as Aldrich Chemical Co. (Milwaukee, WI, USA); Sigma Chemical Co. (St. Louis; MO, USA); and Fluka Chemical Corp. (Ronkonkoma, NY, USA). Methods useful for derivatization of the amino acids identified herein are disclosed in U.S. Serial No. 08/438,644, filed May 10, 1995; U.S. Serial No. 08/372,208, filed January 13, 1995; and PCT/US96/00871, filed January 16, 1996, published July 18, 1996 under International Publication Number WO96/21464

The preferred naturally occurring amino acids used for derivatization to produce the delivery agents used in the methods and compositions hereof in the present invention are alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, hydroxyproline, γ -carboxyglutamate, phenylglycine, or o-phosphoserine. It is particularly desirable to utilize arginine, leucine, lysine, phenylalanine, tyrosine, tryptophan, valine, or phenylglycine as substrates.

The preferred non-naturally occurring amino acids which may be derivatized for use as delivery agents in the present invention are β -alanine, α -amino butyric acid, γ -amino butyric acid, γ -(aminophenyl) butyric acid, α -amino isobutyric acid, 6-aminocaproic acid, 7-amino heptanoic acid, β -aspartic acid, aminobenzoic acid, aminophenyl acetic acid, aminophenyl butyric acid, γ -glutamic acid, cysteine (ACM), ϵ -lysine, ϵ -lysine, methionine sulfone, norleucine, norvaline, ornithine, d-ornithine, p-nitro-phenylalanine, hydroxy proline, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid and thioproline.

Although the present invention encompasses the use of delivery agents prepared by derivatization of any of the amino acids discussed above, preferred derivatized amino acids to employ in the methods and formulations of the present invention are those of Formulas I and II above. Particularly preferred derivatized amino acids useful as delivery agents are those referred to above and in Figure 1 as E352; E94; E198; E702; E597; E445; E579; E594; and E623.

Adjuvants

Adjuvants which assist in inducing tolerance include lipopolysaccharides (LPS) and cholera toxin β -subunit.

Pharmaceutical Formulations

Delivery of pharmaceutical formulations comprised of an antigen and a delivery agent (with or without an adjuvant), with the delivery agent or agents described herein are, preferably a derivatized amino acid of Formulas I and II administered in a dose of about 100-1,000 mg/kg of body weight, results in the induction of oral tolerance.

In one embodiment of the present invention, the derivatized amino acids or salts thereof may be used as delivery agents by simply mixing one or more derivatized amino acids or salts thereof with the antigen (with or without adjuvant) prior to oral administration. In another embodiment, the derivatized amino acids or salts thereof may be used to form microspheres or microcapsules containing the antigen (with or without adjuvant).

Microspheres containing antigen with or without adjuvant can generally be of the matrix form or the microcapsule form. The matrix form includes both a hollow matrix sphere in which the delivery agent forms a matrix shell around a hollow center with the antigen (with or without adjuvant) distributed throughout the matrix and a solid matrix sphere in which the delivery agent forms a spherical matrix continuum in which the antigen (without or without adjuvant) is distributed. The microcapsule form, on the other hand, is one in which the encapsulated antigen (either with or without adjuvant) is either in solution or in solid form, with the delivery agent forming a shell around the encapsulated material.

The formulations of the present invention may also include one or more enzyme inhibitors. Such enzyme inhibitors include, but are not limited to, compounds such as actinonin, aprotinin or epiactinonin and derivatives thereof. Derivatives of these compounds are disclosed in U.S. Patent No. 5,206,384 and are described in the above-identified U.S. Patent Applications Serial Nos. 08/438,644 and 08/372,208; and in PCT/US96/00871, International Publication Number WO96/21464.

The formulations of the present invention may be formulated into oral dosage units by the addition of one or more excipients, diluents, disintegrants, lubricants, plasticizers, colorants, or dosing vehicles. Preferred oral unit dosage forms include, but are not limited to,

tablets, capsules, or liquids. The oral unit dosage forms can include biologically effective amounts of the antigen (with or without a biologically effective amount of an adjuvant) but can include less than such amounts if multiple unit dosage forms are to be used to administer a total dosage of the antigen with or without adjuvant. Oral unit dosage forms are prepared by methods conventional in the art.

The delivery agents of the present invention do not alter the physiological and biological properties of the antigen or the adjuvant. Furthermore encapsulation, if used, need not permanently alter the structure of the antigen.

Inducing Oral Tolerance To Autoimmune Antigens

Also provided herein is a demonstration that the delivery agents of the invention are capable of promoting suppression of EAE through oral tolerance. EAE is widely used as a model for human autoimmune inflammatory demyelinating diseases. (See BACKGROUND above.) It is proposed that the derivatized amino acid delivery agents of the invention act by increasing the fraction of an administered dose of MBP that is absorbed across the GI epithelia. This is very significant, since tolerance is known to be a highly dose-dependent phenomenon. The presence of delivery agents may also lead to a decrease in the variability in responses that accompanies normal GI absorption. The invention thus provides for modulation of immunogenic response, and thus clinical disease, by oral administration of autoantigens accomplished in the presence of delivery agents using smaller or fewer administered doses than are required with the antigen alone. It will be understood that the methods and compositions of the invention are not limited by the foregoing possible mode of action.

EXAMPLES

The following examples are intended to illustrate the invention, without limiting its scope.

Example 1

Single Dose Oral Administration of Sheep Red Blood Cells with Delivery Agent

Five female BALB/c mice were fed a single dose suspension of 2.5×10^9 sheep red blood cells (SRBC) + E94 (600 mg/Kg) in Phosphate Buffered Saline (PBS), 0.1 M phosphate and 0.15 M sodium chloride, pH 7.2. Seven days after completion of oral dosing, mice were primed by footpad injection of 1×10^7 SRBC. They were bled on day 14. On day 21 the mice were tested in a Delayed Type Hypersensitivity (DTH) test through challenge by injection in the footpad not previously used in priming with 1×10^8 SRBC. Footpad thickness was measured before and 24 hours after challenge using a Vernier caliper. On day

28 they were bled again. Sera were placed into Eppendorf tubes and assayed for anti-SRBC IgM (days 14 and 28) and anti-SRBC IgG (day 28) by the direct and indirect hemagglutination assays, respectively. (See assay description below.) Prior to assaying, the samples were heat inactivated at 56°C for 60 minutes. Assay data for Example 1 are found in
 5 Figure 2.

Direct hemagglutination assay for anti-SRBC serum IgM antibodies:

- a) Dilute 10 μ of sera with 190 μ of PBS, pH 7.2 (1/20)
- b) On a rigid, U-bottom microtiter plate, mark wells across the rows for dilutions of samples 40; 80; 160;...81,920
- 10 c) Place 50 μ of PBS in each well
- d) In the first well of duplicate rows, add 50 μ of heat-inactivated, 1/20 diluted sera (first well = 1/40)
 Leave two rows for controls:
 Mixture of IgM + IgG positive controls (Rabbit anti-SRBC IgM and Rabbit anti-SRBC
 15 IgG), 25 μ each diluted 1/20 in PBS
- e) Serially 2x dilute the sera or controls (50 μ) across each row
- f) Add 50 μ of 0.5% SRBC to each well
- g) Cover with pressure-sensitive adhesive plate sealer (COSTAR™) Shake briefly at low speed to mix
- 20 h) Incubate overnight at room temperature, taking care not to disturb the plate at all
- i) Carefully examine for IgM hemagglutination patterns. A positive response appears as a uniform coating of cells adhering to the bottom of wells. A negative response appears as a tight button of settled cells that will stream down if the plate is tilted.
- j) Record average of duplicates.
- 25 k) To assay for anti-SRBC IgG antibodies DO NOT empty wells. Go on to perform indirect HA assay on the same plate.

Indirect hemagglutination assay

for non-agglutinating anti-SRBC serum IgG antibodies:

- a) In a 15 ml tube, add 11 μ of goat anti-mouse IgG (Fc, specific) (2.3 mg/ml) to 5.05 ml
 30 PBS (1/460 dilution; approximate amount needed per plate)
 In a second tube, add 4 μ of goat anti-rabbit IgG (Fc, specific) to 1.8 ml of PBS
 approximate amount needed (per plate)
- b) Add 50 μ of diluted goat anti-mouse IgG (Fc, specific) to the sample wells (0.25 μ g per well)

- Add 0.25 mg of goat anti-rabbit IgG (F_c specific) to each control well
- c) Cover with pressure-sensitive adhesive plate sealer (COSTAR™)
 - d) Shake slowly and briefly to mix
 - e) Incubate 2 hours at room temp. followed by overnight at 4°C
 - 5 f) Carefully examine for IgG hemagglutination titers
 - g) Record average of duplicates.

Comparative Example A

Single Dose Oral Administration of SRBC Alone

- Five female BALB/c mice were fed a single dose suspension of SRBC alone
10 with no delivery agent precisely as described in Example 1. The mice were bled and assayed as described in Example 1. Assay data for Comparative Example A are found in Figure 2.

Example 2

Oral Administration of Sheep Red Blood Cells For Five Consecutive Days With Delivery Agent

- 15 Five female BALB/c mice were fed a suspension of 2.5×10^9 sheep red blood cells (SRBC) + E94 (600 mg/Kg) in Phosphate Buffered Saline (PBS) for five consecutive days. The mice were bled and assayed as described in Example 1. Assay data for Comparative Example B are found in Figure 2.

Comparative Example B

Administration of SRBC Alone For Five Consecutive Days

- 20 Five female BALB/c mice were fed a suspension of SRBC alone with no delivery agent for five consecutive days as described in Example 2. The mice were bled and assayed as described in Example 1. Assay data for Comparative Example B are found in Figure 2.

Comparative Example C

Administration of SRBC Alone For Fifteen Consecutive Days

- 25 Five female BALB/c mice were fed a suspension of SRBC alone with no delivery agent for fifteen consecutive days as described in Comparative Example B. The mice were bled and assayed as described in Example 1. Assay data for Comparative Example
30 B are found in Figure 2.

Comparative Example D

Single Dose Oral Administration of Saline Alone (no SRBC)

Five female BALB/c mice were fed a single oral dose of saline solution with no delivery agent as an unfed control. The mice were bled and assayed as described in

5 Example 1. Assay data for Comparative Example D are found in Figure 2.

* * * * *

As can be seen in Figure 2 (Examples 1 and 2; and Comparative Examples A, B, C and D) a single dose of SRBC in the presence of E94 delivery agent (Example 1) suppressed IgM on Day 14 relative to unfed control (Comparative Example D) significantly
10 more than without delivery agent (Comparative Example A), and even lower than 15 doses of SRBC alone (Comparative Example C). On Day 28, IgM for Example 1 (E94 + SRBCx1) was still lower than that for the Comparative Example D control (90% significance) while no other group was significantly different from the Comparative Example D control.

Figure 2 also shows that for IgG, on Day 28, the Example 1 (E94 + SRBCx1)
15 group was lower than the Comparative Example D control (90% significance) while no other groups were significantly different from the Comparative Example D control.

* * * * *

Example 3

Oral Administration of Influenza Antigen with Delivery Agents After Priming

20 Eight OF-1 female mice were primed subcutaneously with a low dose (5 µg per mouse) of vaccine alone (to mimic a non-naive population) on day 0, followed by oral dosing on day 21 with 60 µg of Influenza antigen per mouse in solution with 750 mg/kg of E94. Sera were collected every two weeks, pooled, and assayed for hemagglutination
25 inhibition. (See assay description below.) Assay data for Example 3 are found in Figure 3.

Hemagglutination Inhibition Assay For Anti-HA Antibodies:

A. Hemagglutination assay to determine virus HA titer

- 1) Use hard plastic-U-bottom plates.
- 2) Mark wells 1-10 as 1/10, 1/20...to 1/5120. Mark #12A and 12B as controls
- 30 3) In a tube, dilute virus suspension 1/10 with PBS.
- 4) Add 50 µl PBS to each well from #2 to #10 in duplicate rows.
- 5) Add 100 µl of diluted virus suspension to well #1.
- 6) Serially 2x dilute 50 µl of the virus across plate until well #10, mixing 7x per dilution.

- 7) Add 50 μ l of well-suspended 0.5% chicken (or sheep) red blood cells to each well (including control).
- 8) Cover plate with sealer.
- 9) Shake briefly on titer plate shaker.
- 5 10) Incubate without shaking at room temperature for 30 minutes.
- 11) Be sure that control wells show a negative pattern (compact settled drip). If not, wait until they do so.
- 12) Immediately note the patterns in each well.
- 13) Record the HA titer of the virus as the highest dilution which resulted in complete agglutination. If duplicates differ by one dilution, take the average. If they differ by more than one dilution, repeat the assay.
- 10 14) This titer provides the dilution of virus suspension which contains one HA unit per 50 μ l. Divide this titer by 8 to get the dilution which will contain 4 HA units per 0.025 μ l for the actual Hemagglutination Inhibition (HI) assay.
- 15 On the day of the HI assay, prepare just enough diluted virus for back-titration. If the back-titration assay is satisfactory, dilute enough virus for the HI assay of the sera samples.

B. Hemagglutination Inhibition (HI) assay of sera:

RDE* treat all test sera, reference sera and negative control sera on the day before the HI assay will be done.

- 20 1. RDE* treatment of sera to remove non-specific inhibitors:
 - a) Reconstitute RDE (Accurate Chemical and Scientific Corp., Westbury, N.Y.) immediately before use.
 - b) Add 100 μ l serum and 300 μ l RDE to 2 ml Eppendorf tube Vortex briefly
 - c) Incubate at 37/C overnight
 - 25 d) Prepare 2.5% sodium citrate solution: 2.5 g sodium citrate plus distilled water q.s. to 100 ml
 - e) Add 300 μ l sodium citrate solution to the sample tube
 - f) Incubate at 56/C for 30 minutes
 - g) Add 300 μ l of PBS. This will result in a 1/10 starting dilution of serum.

30 2. HI assay:

A. Back-titration of virus:

- a) In a hard plastic plate, add 100 μ l of PBS to duplicate wells 1-5 (rows A and B) and to two control wells

- b) Add 50 μ l of the diluted virus suspension (part A #15 above) to well 1 of each row
- c) Serially 2x dilute across to wells A5 and B5
- d) Add 50 μ l of 0.5% SRBC to all wells, including the control wells
- e) Cover, shake briefly, and incubate 30 minutes
- 5 f) The first 3 wells should be completely agglutinated, 4 and 5 should be partially or not at all agglutinated. If this is not the case, the virus stock should be diluted appropriately to correct for this difference and re-assayed.

B. HI assay:

- 10 1. Use flexible plates. Mark columns for duplicate dilution series of each test and control serum sample (normal naive serum and positive reference anti-serum). The plate should have 11 columns per row for dilutions plus column 12 for RBC alone.
- 2. To all wells, except column 1, add 25 μ l of PBS.
- 3. To column 1 wells, add 50 μ l of the appropriate RDE-treatment serum (test, positive, or negative control) sample.
- 15 4. Serially 2x dilute 25 μ l of the sera across to column 11, mixing 7x per dilution.
- 5. Add 25 μ l of diluted virus (containing 4 HA units per 25 μ l) to all serum dilution wells, columns 1-11. Add 25 μ l of PBS to column 21.
- 6. Cover plate, mix briefly on shaker, and incubate at r.t. for 30 minutes.
- 7. Add 50 μ l of well-suspended 0.5% red blood cells to all wells, including the RBC control wells (column 12). Cover and shake briefly.
- 20 8. Incubate at room temperature for 30-45 minutes, until RBC controls show a compact, negative pattern.
- 9. Read patterns immediately.
- 10. Negative control (naive) serum: all wells should show complete agglutination (i.e. no inhibition since there is no anti-HA antibody).
- 25 11. Positive control (reference) serum; should see uniform inhibition in the dilution series up to the theoretical titer of the reference serum.

Comparative Example E

Single Dose Oral Administration of Influenza Antigen Alone

- 30 Eight OF-1 female mice were treated and fed a single dose preparation of influenza antigen alone with no delivery agent as described in Example 3. The mice were bled and assayed as described in Example 3. Assay data for Comparative Example E are found in Figure 3.

As can be seen in Figure 3 (Example 3 and comparative Example E) a single dose of influenza antigen with E94 delivery agent (Example 3) suppressed the production of anti-influenza antibodies relative to control fed only influenza antigen (Comparative Example E) by more than 2 fold.

- 5 Virtually all adult humans have been exposed to Influenza at one or more times. Thus, most people have some levels of pre-existing immunity to Influenza. This pre-existing condition will influence their susceptibility to infection to cross-reacting strains of the disease. To simulate the effects of immunization with oral vaccine in this non-naïve population, the mice were pre-immunized with a lower dose of antigen than would be
10 required to fully immunize them.

Example 4

Oral Administration of Influenza Antigen With Delivery Agents

- Ten BALB/c mice were administered a single oral dose of 15 µg of influenza antigen [influenza vaccine A/Johannesburg/39/94 (H3N2)] per mouse with 500 mg/kg of
15 E352. Sera were collected every two weeks, pooled, and assayed for anti-hemagglutinin (HA) IgG. (See assay description below.) Assay data for Example 4 are found in Figure 4.

ELISA For Determining Anti-HA IgG Isotype Antibodies in Serum:

1. Coat plates with HA antigen, 10 µg/mL in carbonate buffer.
2. Wash x 4.
- 20 3. Block with Superblock™ or 1/10 diluted Bovine Serum Albumin (BSA).
4. Add 100 µL per well of diluent (Superblock™ or 1/15 diluted BSA) to all except the top row.
5. In the first row, add 150 µL per well of 1/100 diluted test serum in duplicate and serially dilute 3x down the plate. Leave 2 empty wells as background.
- 25 6. Incubate 2 hours at room temperature.
7. Wash x 8.
8. To each well, add 100 µl of IgG isotype-specific anti-mouse alkaline-phosphatase conjugated antibody (diluted with 4% PEG 6000).20. Incubate overnight at 4/C.
9. Wash x 8.
- 30 10. Add 100 µL per well of p-nitrophenyl phosphate (pNPP) substrate solution and incubate in the dark with shaking for 30 minutes.
11. Read and record OD₄₀₅ after subtracting the background absorbance.

Comparative Example F

Oral Administration of Influenza Antigen Alone

Ten BALB/c mice were fed a single dose preparation of influenza antigen [influenza vaccine A/Johannesburg/39/94 (H3N2)] alone with no delivery agent as described in Example 4. The mice were bled and assayed as described in Example 4. Assay data for Comparative Example F are found in Figure 4.

As can be seen in Figure 4 (Example 4 and Comparative Example F) a single dose of influenza antigen with E352 delivery agent (Example 4) significantly suppressed the production of anti-influenza antibodies relative to control fed only influenza antigen.

Example 5

Two Dose Oral Administration of Ovalbumin with Delivery Agent

A stock solution was prepared by dissolving Ovalbumin, 10 mg/ml, in 10 mM phosphate buffer (pH 7.4). This solution was diluted with buffer to provide 1.0 mg in a volume of 0.2 ml. Ten BALB/c female mice were administered two oral doses of 1 mg ovalbumin per mouse with 600 mg/kg of E94 delivery agent at weeks 0 and 4.

Systemic challenge was achieved by intramuscular (IM) injection of 2 mg/ml ovalbumin with 50% Complete Freund's Adjuvant (CFA) at week 9. Serum samples were collected at week 12 and assayed for anti-ovalbumin total IgG isotypes as described in Example 4 utilizing ovalbumin antigen instead of HA antigen. Assay data for Example 5 are found in Figure 5.

Comparative Example G

Two Dose Oral Administration of Ovalbumin Alone

Ten BALB/c female mice were fed two oral doses of 1 mg ovalbumin per mouse alone with no delivery agent at weeks 0 and 4 as described in Example 5. The mice were challenged, bled and assayed as described in Example 5. Assay data for Comparative Example G are found in Figure 5.

* * * * *

Figure 5 illustrates the anti-Ova IgG response in mice immunized orally with two doses of 1 mg Ovalbumin each with or without delivery agent E94, four weeks apart. They were then challenged intramuscularly with Ovalbumin in complete Freund's adjuvant. The response to the challenge with antigen alone (Comp. Ex. G) was the same as in mice given the intramuscular dose alone. However, following feeding in the presence of delivery agent (Ex. 5), the response was significantly suppressed compared to both unfed and antigen-alone fed (Comp. Ex. G) animals. This indicates induction of tolerance in the presence of

delivery agent following feeding of a dose which is non-tolerizing in the absence of the delivery agent.

* * * * *

Example 6

5 Single Dose Oral Administration of Ovalbumin with Delivery Agent

A stock solution of ovalbumin was prepared by dissolving Ovalbumin, 125 mg/ml, in 10 mM phosphate buffer (pH 7.4). This solution was used to provide 25 mg in a volume of 0.2 ml. Five BALB/c mice were administered a single oral dose of 25 mg ovalbumin per mouse with 600 mg/kg of E94 delivery agent.

10 Challenge was achieved by subcutaneous (SC) injection of 0.1 mg ovalbumin with 50% Complete Freund's Adjuvant (CFA) at week 1. Serum samples were collected at week 4 and assayed for anti-ovalbumin total IgG isotypes as described in Example 5. Assay data for Example 6 are found in Figure 6.

Comparative Example H

15 Oral Dose Administration of Ovalbumin Alone

Five BALB/c female mice were fed a single oral dose of 25 mg ovalbumin per mouse alone with no delivery agent as described in Example 6. The mice were challenged, bled and assayed as described in Example 6. Assay data for Comparative Example E are found in Figure 6.

20 Comparative Example I

Unfed Mice for Control

Five BALB/c female mice were fed nothing for use as a control. The mice were challenged, bled and assayed as described in Example 5 for data described in Figure 5. The mice were challenged, bled and assayed as described in Example 6 for data described in
25 Figure 6.

* * * * *

Figure 6 illustrates that animals fed 25 mg Ovalbumun with delivery agent E94 and then challenged subcutaneously one week later with Ovalbumin in complete Freund's adjuvant show significantly suppressed serum anti-Ova IgG titers than those which were not pre-fed.
30 While mice fed antigen alone were also suppressed (Comp. Ex. H), this suppression was not statistically significant, while that induced in the presence of E94 (Ex. 6) was significant. Thus, E94 allowed a more consistent suppression of antibody induction than the antigen alone.

* * * * *

Example 7

Single Dose Oral Administration of Sheep Red Blood Cells with Delivery Agent

5 Five female BALB/c mice were fed a single dose suspension of 2.5×10^9 sheep red blood cells (SRBC) + E594 (600 mg/Kg) in Phosphate Buffered Saline (PBS), 0.1 M phosphate and 0.15 M sodium chloride, pH 7.2. Seven days after completion of oral dosing, mice were primed by footpad injection of 1×10^7 SRBC. They were bled on day 14. Sera were placed into Eppendorf tubes and assayed for anti-SRBC IgG (day 14) by the
10 indirect hemagglutination assays. (See assay description in Example 1 above.) Prior to assaying, the samples were heat inactivated at 56°C for 60 minutes. Assay data for Example 7 are found in Figure 7.

Comparative Example J

Single Dose Oral Administration of SRBC Alone

15 Five female BALB/c mice were fed a single dose suspension of SRBC alone with no delivery agent as described in Example 7. The mice were bled and assayed as described in Example 7. Assay data for Comparative Example J are found in Figure 7.

Comparative Example K

Single Dose Oral Administration of Saline Alone (no SRBC)

20 Five female BALB/c mice were fed a single oral dose of saline solution with no delivery agent as an unfed control. The mice were bled and assayed as described in Example 7. Assay data for Comparative Example K are found in Figure 7.

* * * * *

25 As can be seen in Figure 7 (Example 7 and Comparative Examples J and K) a single dose of SRBC in the presence of E594 delivery agent (Example 7) suppressed IgG on Day 14 relative to unfed control (Comparative Example K) significantly more than without delivery agent (Comparative Example J).

* * * * *

Example 8**Single Dose Oral Administration of
Sheep Red Blood Cells with Delivery Agent**

Five female BALB/c mice were fed a single dose suspension of 2.5×10^9 sheep red blood cells (SRBC) + E594 (600 mg/Kg) as described in Example 7. Seven days after completion of oral dosing, mice were primed by footpad injection of 1×10^7 SRBC. On day 14, footpad thickness was measured according to the Delayed Type Hypersensitivity (DTH) method outlined in Example 1. The DTH data for Example 8 are found in Figure 8.

Example 9**Single Dose Oral Administration of
Sheep Red Blood Cells with Delivery Agent**

Five female BALB/c mice were fed a single dose suspension of 2.5×10^9 sheep red blood cells (SRBC) + E198 (600 mg/Kg) and tested for footpad thickness (DTH) as described in Example 8. The DTH data for Example 9 are found in Figure 8.

Comparative Example L**Single Dose Oral Administration of Saline Alone (no SRBC)**

Five female BALB/c mice were fed a single oral dose of saline solution with no delivery agent as an unfed control and tested for footpad thickness (DTH) as described in Example 8. The DTH data for Comparative Example L are found in Figure 8.

Comparative Example M**Single Dose Oral Administration of SRBC Alone**

Five female BALB/c mice were fed a single dose suspension of SRBC alone with no delivery agent and tested for footpad thickness (DTH) as described in Example 8. The DTH data for Comparative Example M are found in Figure 8.

* * * * *

As can be seen in Figure 8 (Examples 8 and 9 and Comparative Examples L and M) a single dose of SRBC in the presence of E594 delivery agent (Example 8) or E198 delivery agent (Example 9) suppressed the DTH response on Day 14 relative to unfed control (Comparative Example L) significantly more than without delivery agent (Comparative Example M).

* * * * *

Example 10

Single Dose Oral Administration of Ovalbumin with Delivery Agent

A stock solution of ovalbumin was prepared by dissolving Ovalbumin, 10 mg/ml in 10 mM phosphate buffer (pH 7.4). This solution was diluted 2-fold and used to provide 1.0 mg in a volume of 0.2 ml. Five BALB/c mice were administered a single oral dose of 1.0 mg ovalbumin per mouse with 600 mg/kg of E702 delivery agent.

Challenge was achieved by subcutaneous (SC) injection of 0.1 mg ovalbumin with 50% Complete Freund's Adjuvant (CFA) at week 3. DTH assay was performed as described in Examples 1 and 8. Assay data for Example 10 are found in Figure 9.

Comparative Example N

Single Oral Administration of Ovalbumin Alone

Five BALB/c female mice were fed a single oral dose of 1.0 mg ovalbumin per mouse alone with no delivery agent as described in Example 10. The mice were challenged and assayed for DTH as described in Example 10. Assay data for Comparative Example N are found in Figure 9.

Comparative Example O

Unfed Control

Five BALB/c female mice were fed a single oral dose of saline with no delivery agent as an unfed control. The mice were challenged and assayed for DTH as described in Example 10. Assay data for Comparative Example O are found in Figure 9.

Figure 9 illustrates that animals fed 1.0 mg Ovalbumin with delivery agent E702 and then challenged subcutaneously 3 weeks later with Ovalbumin in complete Freund's adjuvant show significantly suppressed response in the DTH than those which were not pre-fed. Mice fed antigen alone at this dosage were not suppressed (Comparative Example N).

Example 11

Use of delivery agents in the MBP/EAE model for oral tolerance induction

Groups of five female Lewis rats were given one or five oral doses of Myelin Basic Protein (MBP, 1.0 mg per dose every 2-3 days) or a single oral dose of 1.0 mg of MBP together with delivery agents E94, E352 or E702. Two days after the (last) oral dose, all

groups were challenged in the footpad with 0.05 mg of MBP emulsified with Complete Freund's Adjuvant containing Mycobacterium tuberculosis H37Ra, 4 mg/ml. Clinical signs of disease were observed starting on Day 11 after the challenge and rated on a scale of 0 (no disease) to 5 (death). Data for Example 11 are provided in Figures 10, 11 and 12.

5

Figures 10 and 11 show the suppression of clinical disease by a single dose of MPB with E94 and E702 (Figure 10) and E352 (Figure 11). The presence of delivery agents suppressed disease symptoms to a degree statistically identical to 5 doses of MBP alone, and significantly more than a single dose of MBP alone at the time points indicated. In addition, the mean day of onset of paralysis (defined as a clinical disease score less than or equal to 1) was delayed from Day 13 after a single dose of MBP alone to Day 15 in the presence of E94 or E702 and Day 16 in the presence of E352.

Figure 12 shows the mean Maximal Score per group and the Mean Disease Index per group (defined as the highest mean score multiplied by the duration of symptoms) for rats dosed with a single dose of MBP and E352 vs. one or five doses of MBP alone. In both cases, the presence of E352 suppressed these disease parameters significantly compared with the dose of MBP alone, and was statistically identical to the five-dose MBP group.

15

The subscripts in the group labels refer to the number of paralyzed rats in each group, and the "% protection", i.e. the percent of animals that were not paralyzed.

We Claim:

1. A method of inducing oral tolerance in an animal, comprising orally administering to said animal a pharmaceutical formulation comprising an antigen and a delivery agent comprising at least one derivatized amino acid or a salt thereof in an amount sufficient to induce oral tolerance.

2. The method of claim 1, wherein the derivatized amino acid is an acylated amino acid compound of the formula



wherein:

Ar is a substituted or unsubstituted phenyl,

R is $\text{NH}(\text{R}')-\text{C}-$

R' is C_1 to C_{10} alkyl, C_1 to C_{10} alkenyl, phenyl, naphthyl, (C_1 to C_{10} alkyl) phenyl, (C_1 to C_{10} alkenyl) phenyl, (C_1 to C_{10} alkyl) naphthyl, (C_1 to C_{10} alkenyl) naphthyl;

R' is optionally substituted with C_1 to C_4 alkyl, C_1 to C_4 alkenyl, C_1 to C_4 alkoxy, $-\text{OH}$, $-\text{SH}$ and $-\text{CO}_2\text{R}^2$, cycloalkyl, cycloalkenyl, heteroalkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof; and

R^2 is hydrogen, C_1 to C_4 alkyl or C_1 to C_4 alkenyl.

3. The method of claim 1, wherein the derivatized amino acid is a sulphonated amino acid compound of the formula



wherein:

Ar is a substituted or unsubstituted phenyl,

R is $\text{NH}(\text{R}')-\text{C}-$

R' is C_1 to C_{10} alkyl, C_1 to C_{10} alkenyl, phenyl, naphthyl, (C_1 to C_{10} alkyl) phenyl, (C_1 to C_{10} alkenyl) phenyl, (C_1 to C_{10} alkyl) naphthyl, (C_1 to C_{10} alkenyl) naphthyl;

R' is optionally substituted with C_1 to C_4 alkyl, C_1 to C_4 alkenyl, C_1 to C_4 alkoxy, $-\text{OH}$, $-\text{SH}$ and $-\text{CO}_2\text{R}^2$, cycloalkyl, cycloalkenyl, heteroalkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof; and

R^2 is hydrogen, C_1 to C_4 alkyl or C_1 to C_4 alkenyl.

4. The method of claim 1, wherein the antigen is selected from the group consisting of synthetic proteins, naturally produced proteins, synthetic peptides, naturally produced peptides, carbohydrates and lipopolysaccharides.

5. The method of claim 1, wherein the antigen is associated with the induction of auto-immune diseases, clinical (allergic) hypersensitivities or allograft rejection, and subunits or extracts therefrom.

6. The method of claim 1, wherein the formulation incorporates the derivatized amino acid at a dose of 100-1000 mg per kg of body weight.

7. The method of claim 6, wherein the formulation incorporates the derivatized amino acid at a dose of 250-750 mg per kg of body weight.

8. The method of claim 1, wherein the formulation additionally incorporates an enzyme inhibitor.

9. The method of claim 1, wherein the formulation additionally incorporates an adjuvant.

10. A pharmaceutical formulation for inducing oral tolerance in a mammal, comprising an antigen and a delivery agent comprising at least one derivatized amino acid or a salt thereof in an amount sufficient to induce oral tolerance.

11. The pharmaceutical formulation of claim 10, wherein the derivatized amino acid is an acylated amino acid compound of the formula



wherein:

Ar is a substituted or unsubstituted phenyl,

R is $\text{NH}(\text{R}^1)-\text{C}-$

R^1 is C_1 to C_{10} alkyl, C_1 to C_{10} alkenyl, phenyl, naphthyl, (C_1 to C_{10} alkyl) phenyl, (C_1 to C_{10} alkenyl) phenyl, (C_1 to C_{10} alkyl) naphthyl, (C_1 to C_{10} alkenyl) naphthyl;

R^1 is optionally substituted with C_1 to C_4 alkyl, C_1 to C_4 alkenyl, C_1 to C_4 alkoxy, $-\text{OH}$, $-\text{SH}$ and $-\text{CO}_2\text{R}^2$, cycloalkyl, cycloalkenyl, heteroalkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof; and

R^2 is hydrogen, C_1 to C_4 alkyl or C_1 to C_4 alkenyl.

12. The pharmaceutical formulation of claim 10, wherein the derivatized amino acid is a sulphonated amino acid compound of the formula



wherein:

Ar is a substituted or unsubstituted phenyl,

R is $\text{NH}(\text{R}^1)-\text{C}-$

R^1 is C_1 to C_{10} alkyl, C_1 to C_{10} alkenyl, phenyl, naphthyl, (C_1 to C_{10} alkyl) phenyl, (C_1 to C_{10} alkenyl) phenyl, (C_1 to C_{10} alkyl) naphthyl, (C_1 to C_{10} alkenyl) naphthyl;

R¹ is optionally substituted with C₁ to C₄ alkyl, C₁ to C₄ alkenyl, C₁ to C₄ alkoxy, -OH, -SH and -CO₂R², cycloalkyl, cycloalkenyl, heteroalkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof; and

R² is hydrogen, C₁ to C₄ alkyl or C₁ to C₄ alkenyl.

13. The pharmaceutical formulation claim 10, wherein the derivatized amino acid is administered at a dose of about 100-1000 mg per kg of body weight.

14. The pharmaceutical formulation claim 10, wherein the derivatized amino acid is administered at a dose of about 250-750 mg per kg of body weight.

15. The pharmaceutical formulation of claim 10, wherein the antigen is selected from the group consisting of synthetic proteins, naturally produced proteins, synthetic peptides, naturally produced peptides, carbohydrates and lipopolysaccharides.

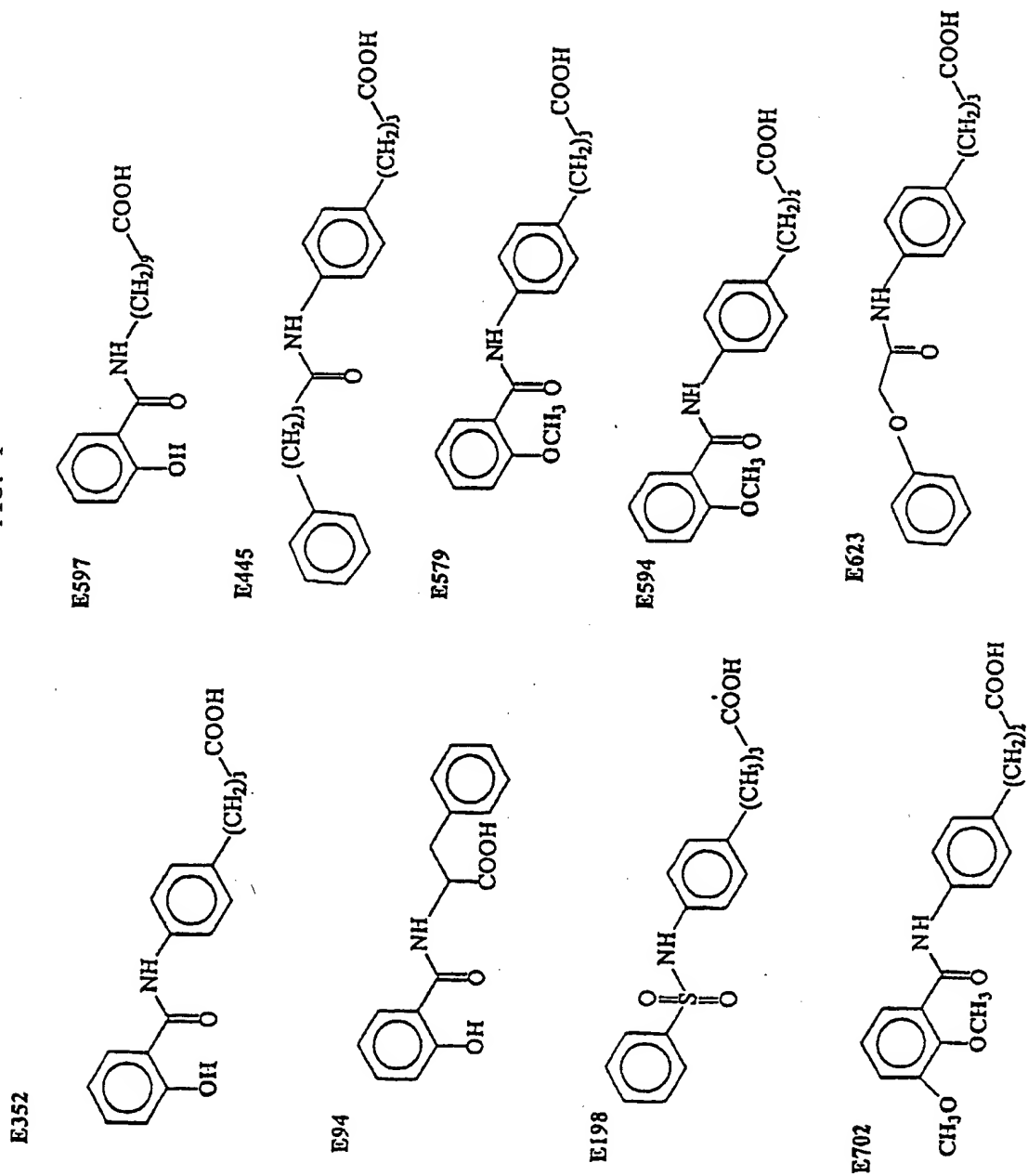
16. The pharmaceutical formulation of claim 10, wherein the antigen is associated with the induction of auto-immune diseases, clinical (allergic) hypersensitivities or allograft rejection, and subunits or extracts therefrom.

17. The pharmaceutical formulation of claim 10, wherein the formulation additionally incorporates an adjuvant.

18. The pharmaceutical formulation of claim 10, wherein the formulation additionally incorporates an enzyme inhibitor.

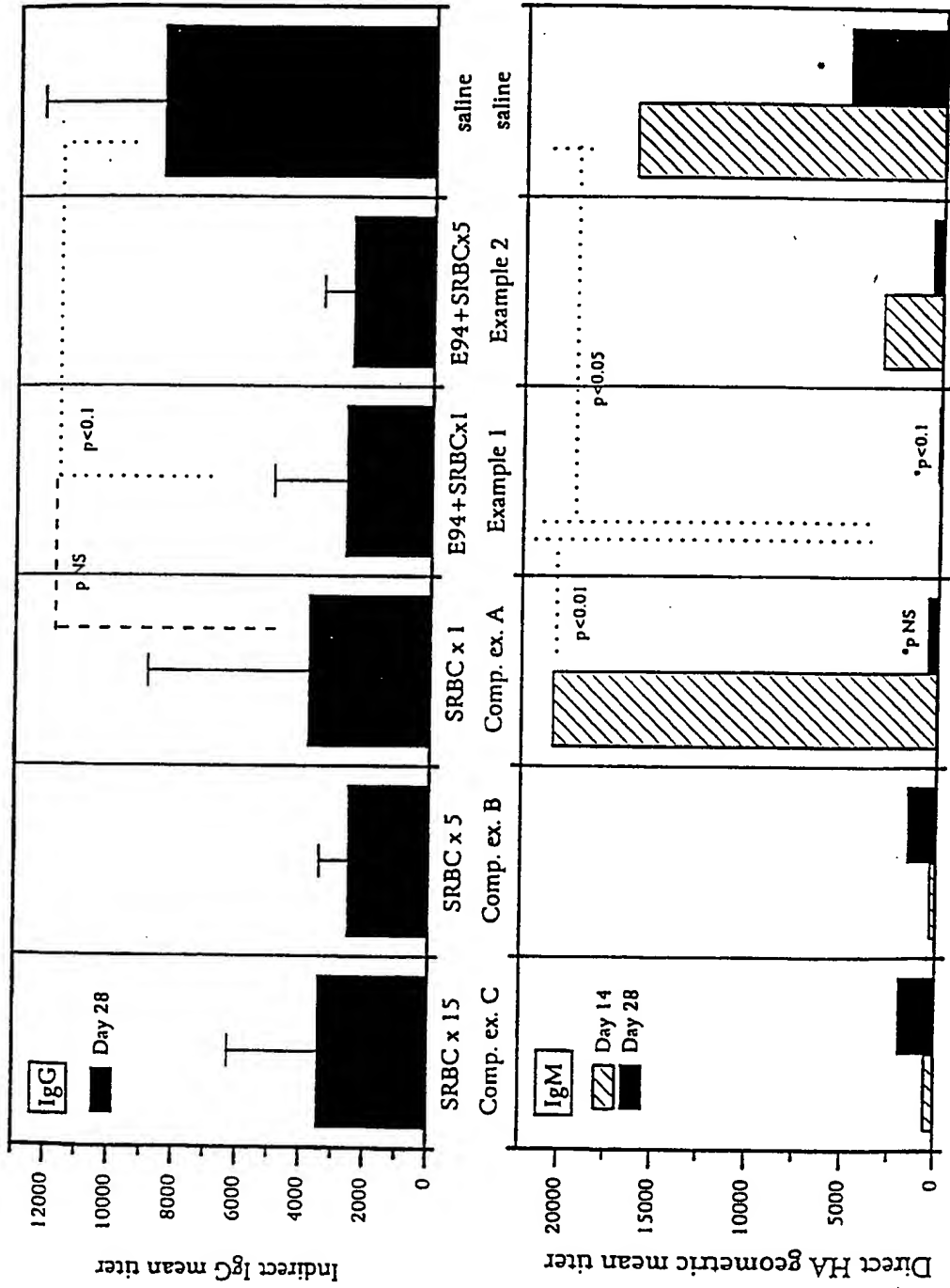
1 / 12

FIG. 1



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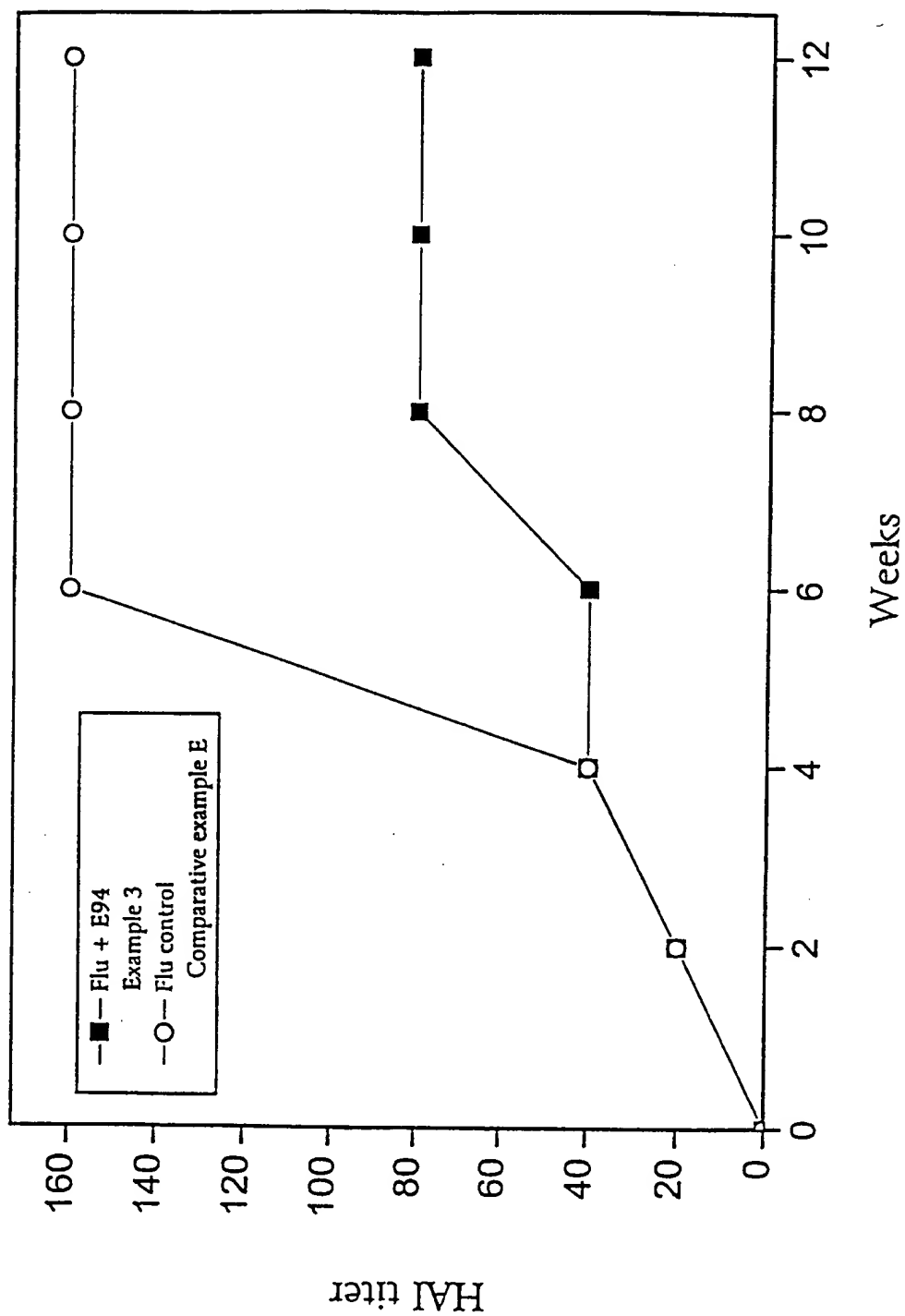
FIG. 2
Serum anti-SRBC antibodies in mice fed SRBC
[Primed (footpad) Day 7
DTH challenge Day 21]



3 / 12

FIG 3

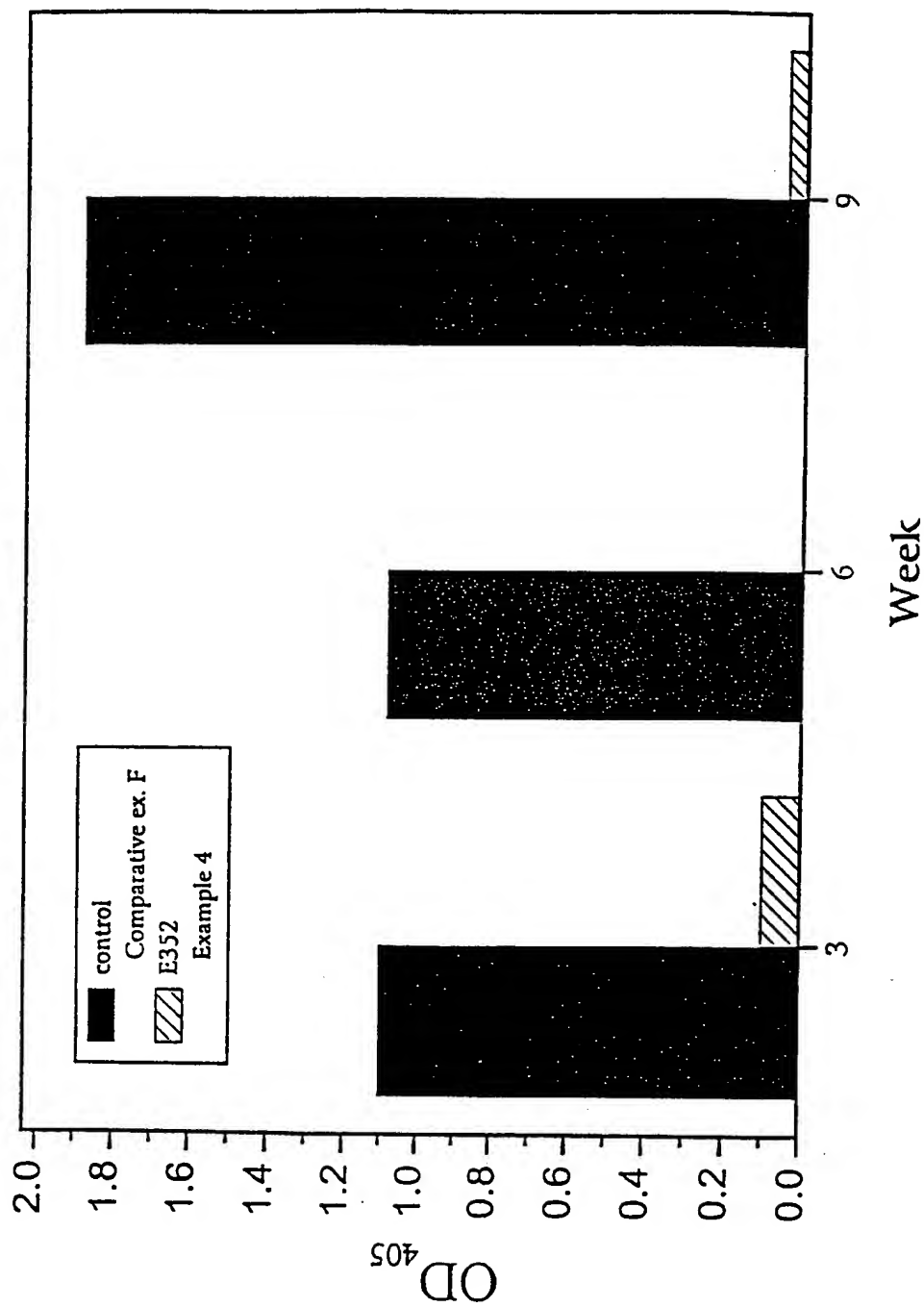
Hemagglutination Inhibition titers of pooled sera.
OF-1 mice pre-immunized subcut. with 5 μ g Influenza antigen
Orally immunized (week 3) with 60 μ g monovalent Influenza antigen
+/- delivery agent E94 in solution



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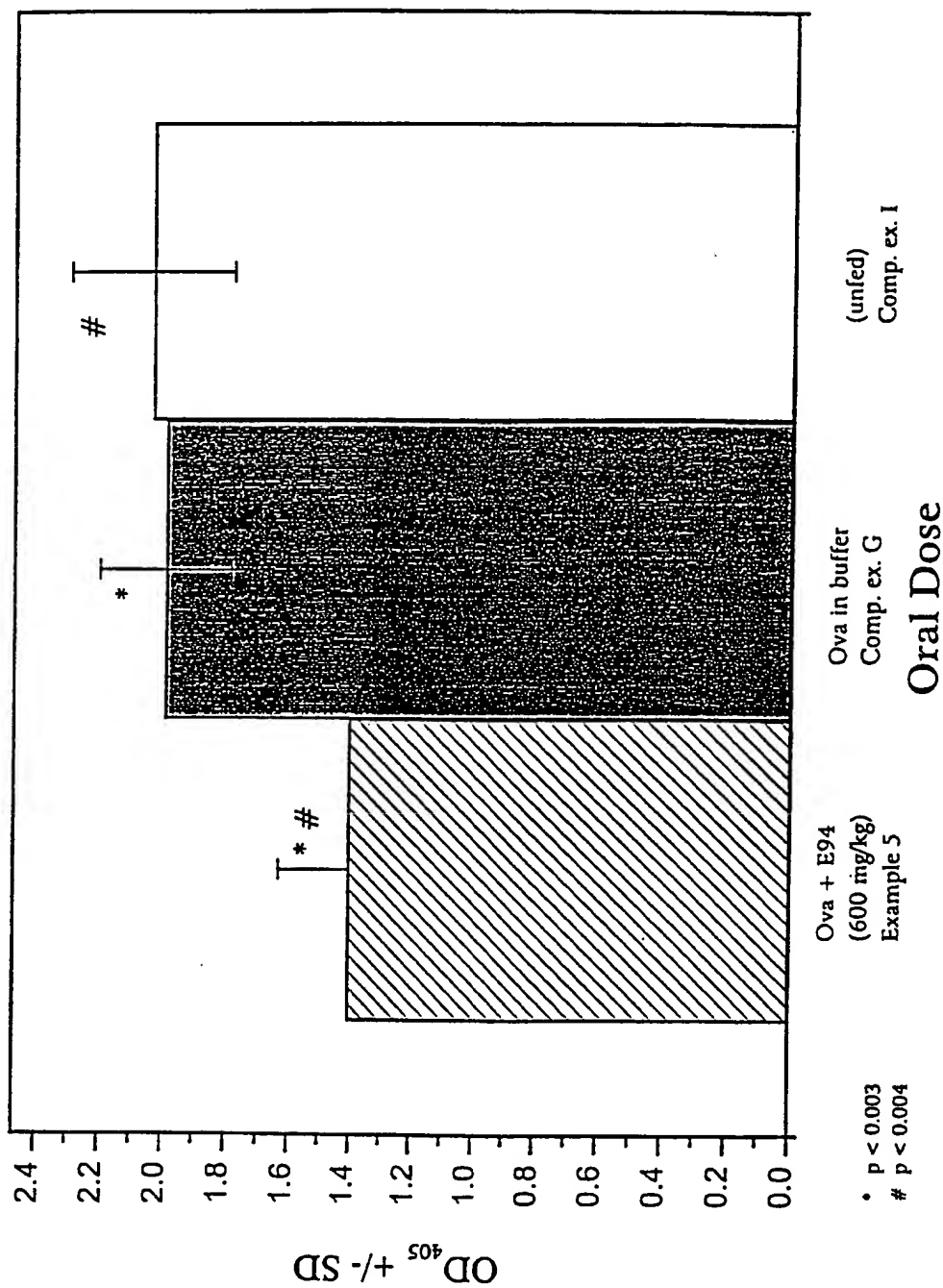
FIG. 4

Serum anti-HA IgG following oral immunization
Single dose Influenza vaccine A/Johannesburg/39/94 (H3N2), 0.75 μ g/kg
+ Emisphere delivery agent E352 (500 mg/kg)



5 / 12

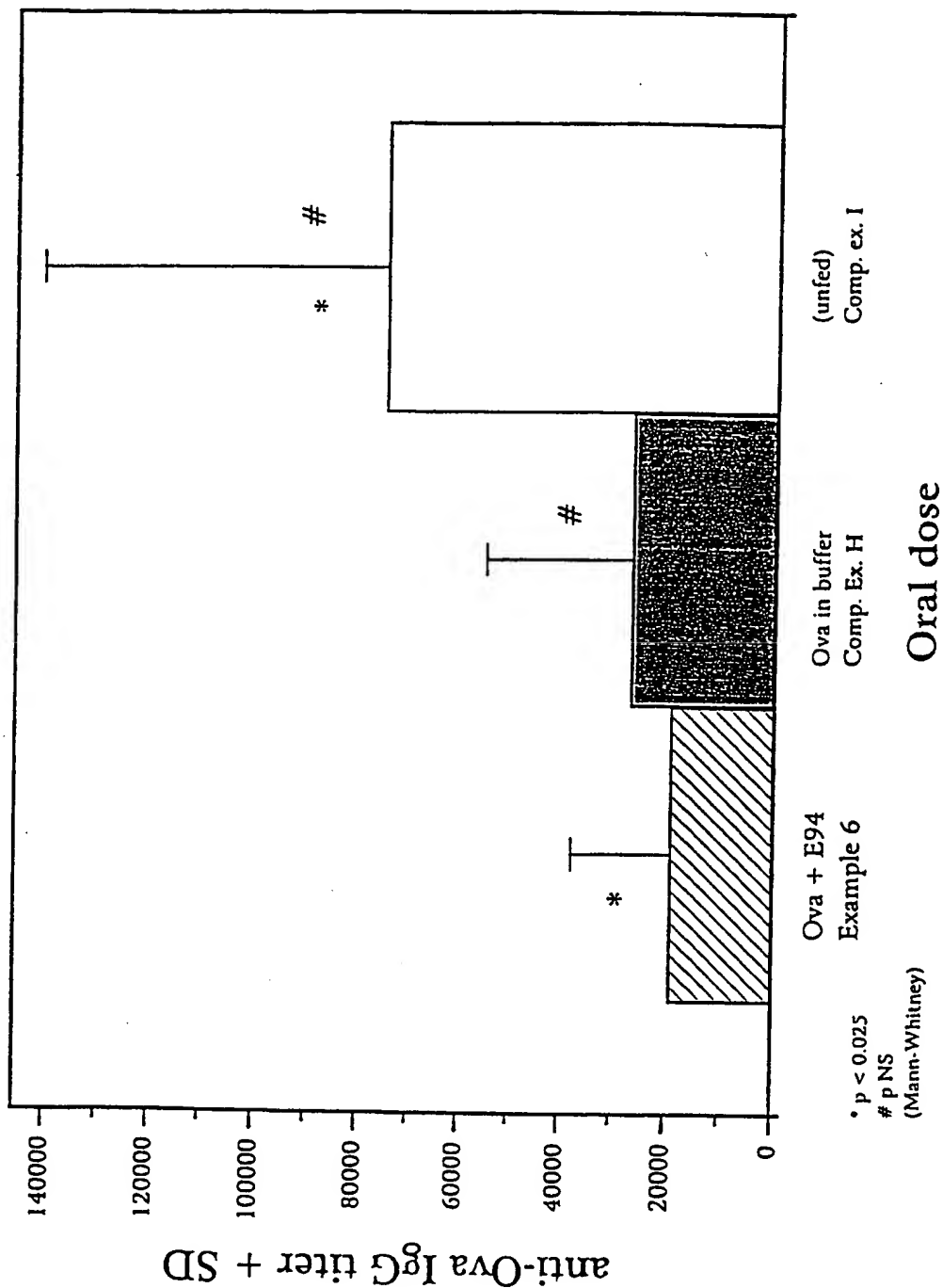
FIG. 5
BALB/c mice orally immunized
weeks 0 and 4, 1.0 mg Ova per dose +/- delivery agent
Systemic (IM) challenge week 9, Ova/CFA.
Serum anti-Ova IgG (week 12)



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FIG. 6

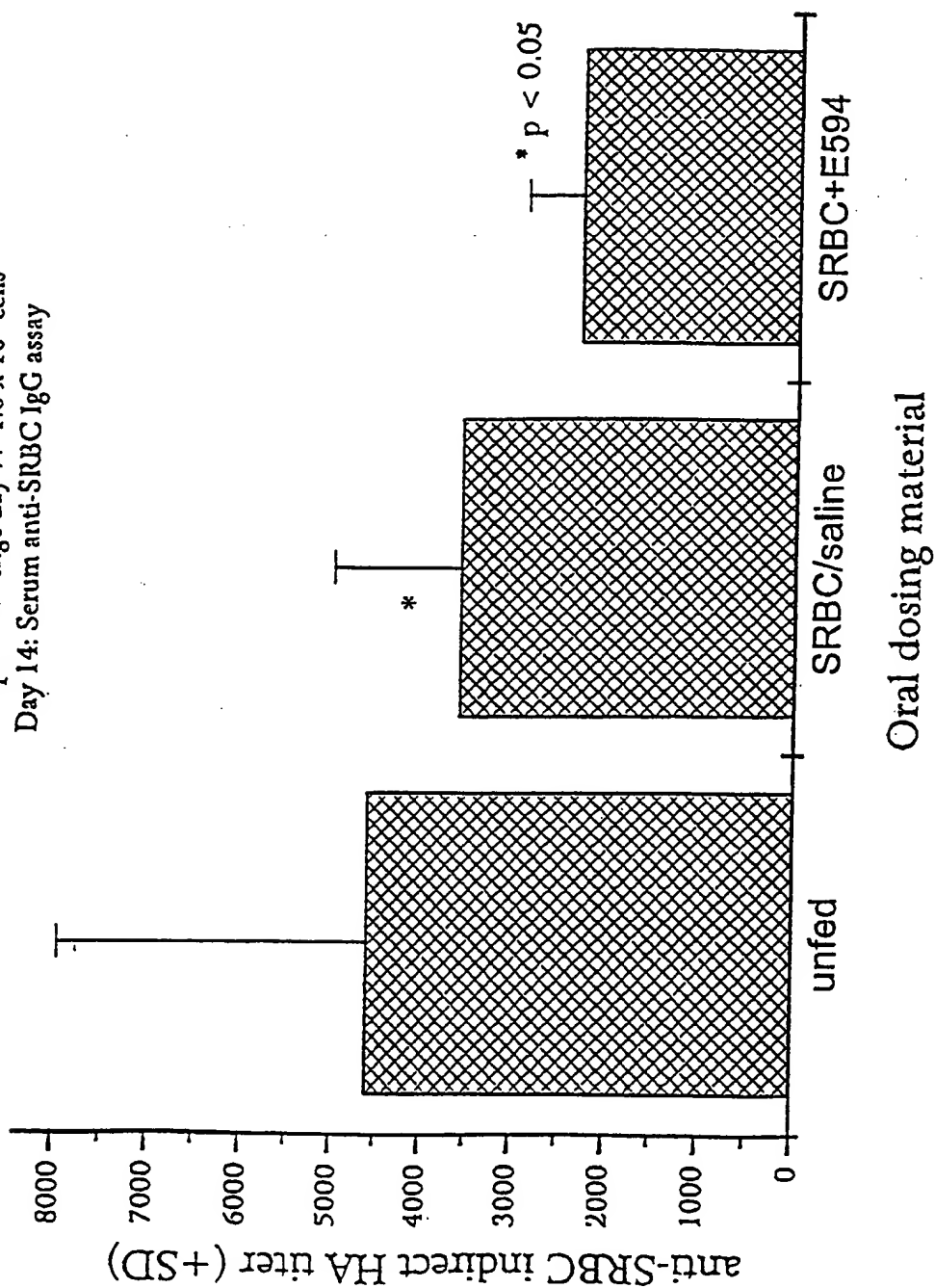
Week 0: gastric administration, 25 mg Ova +/- delivery agent
 Week 1: sc challenge 0.1 mg Ova/CFA
 Serum anti-Ova IgG week 4



7 / 12

FIG. 7

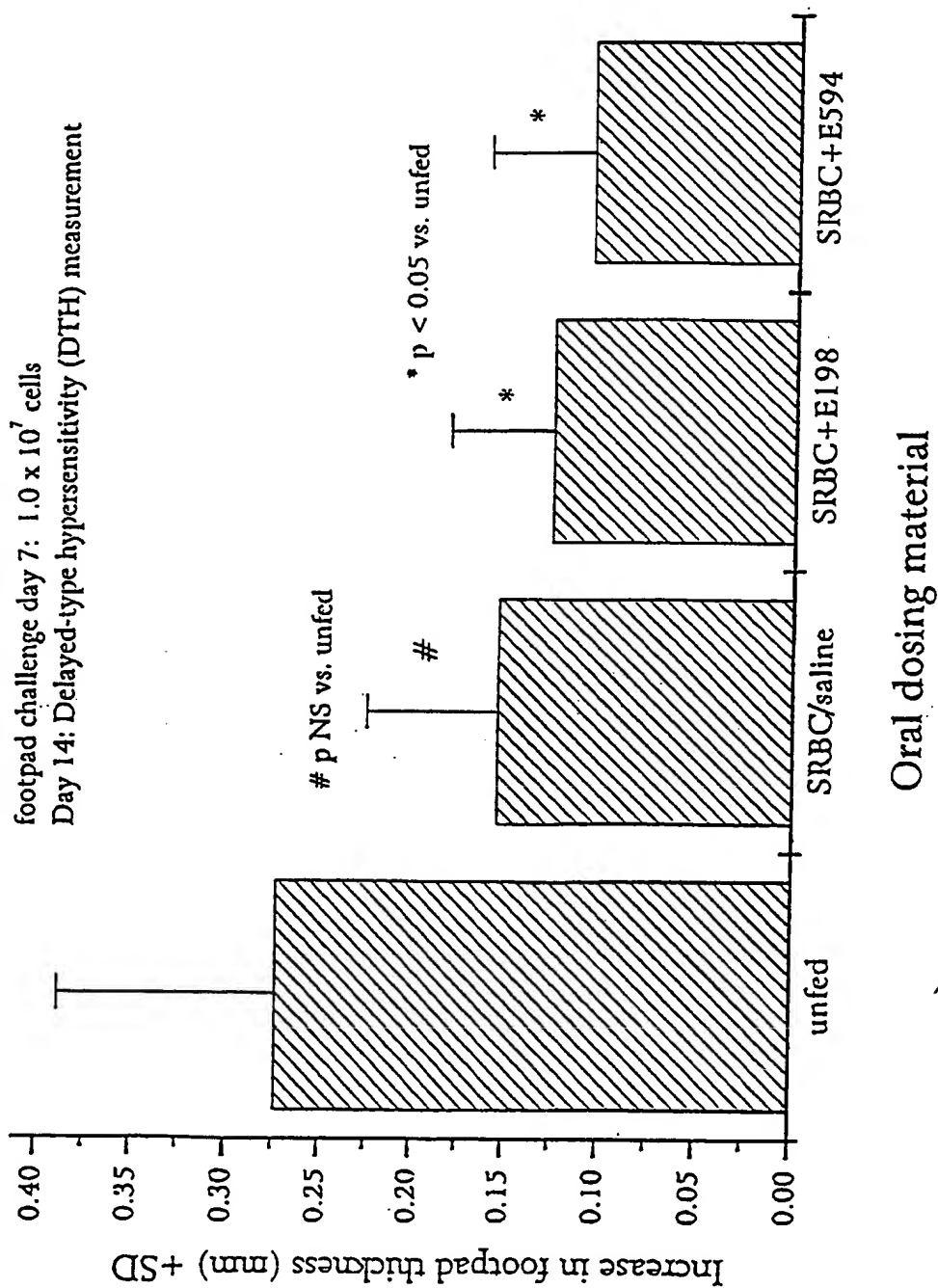
Sheep Red Blood Cells (SRBC)
Single oral dose 2.5×10^9 cells
footpad challenge day 7: 1.0×10^7 cells
Day 14: Serum anti-SRBC IgG assay



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FIG. 8

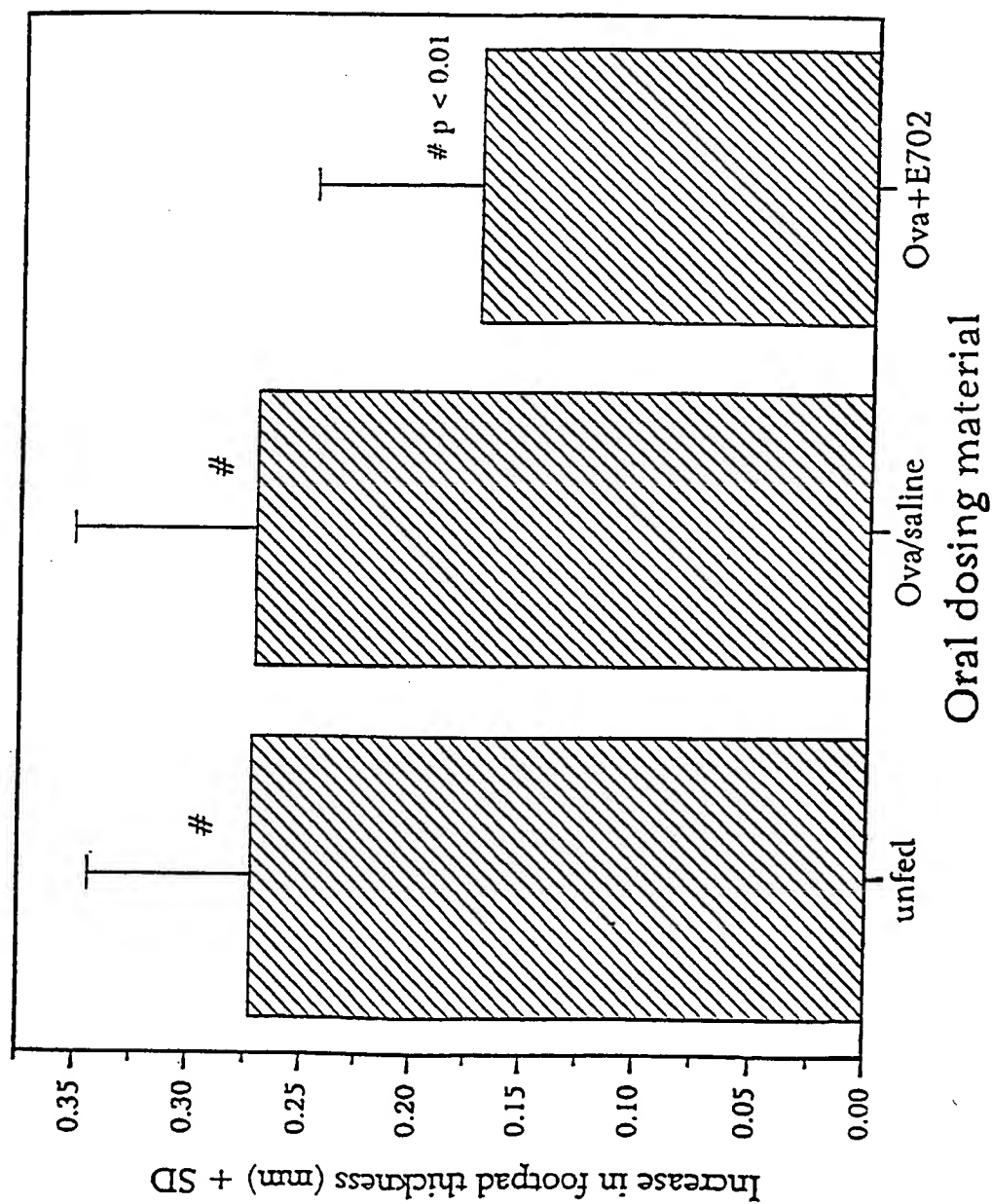
Sheep Red Blood Cells (SRBC)
Single oral dose 2.5×10^9 cells
footpad challenge day 7: 1.0×10^7 cells
Day 14: Delayed-type hypersensitivity (DTH) measurement



9 / 12

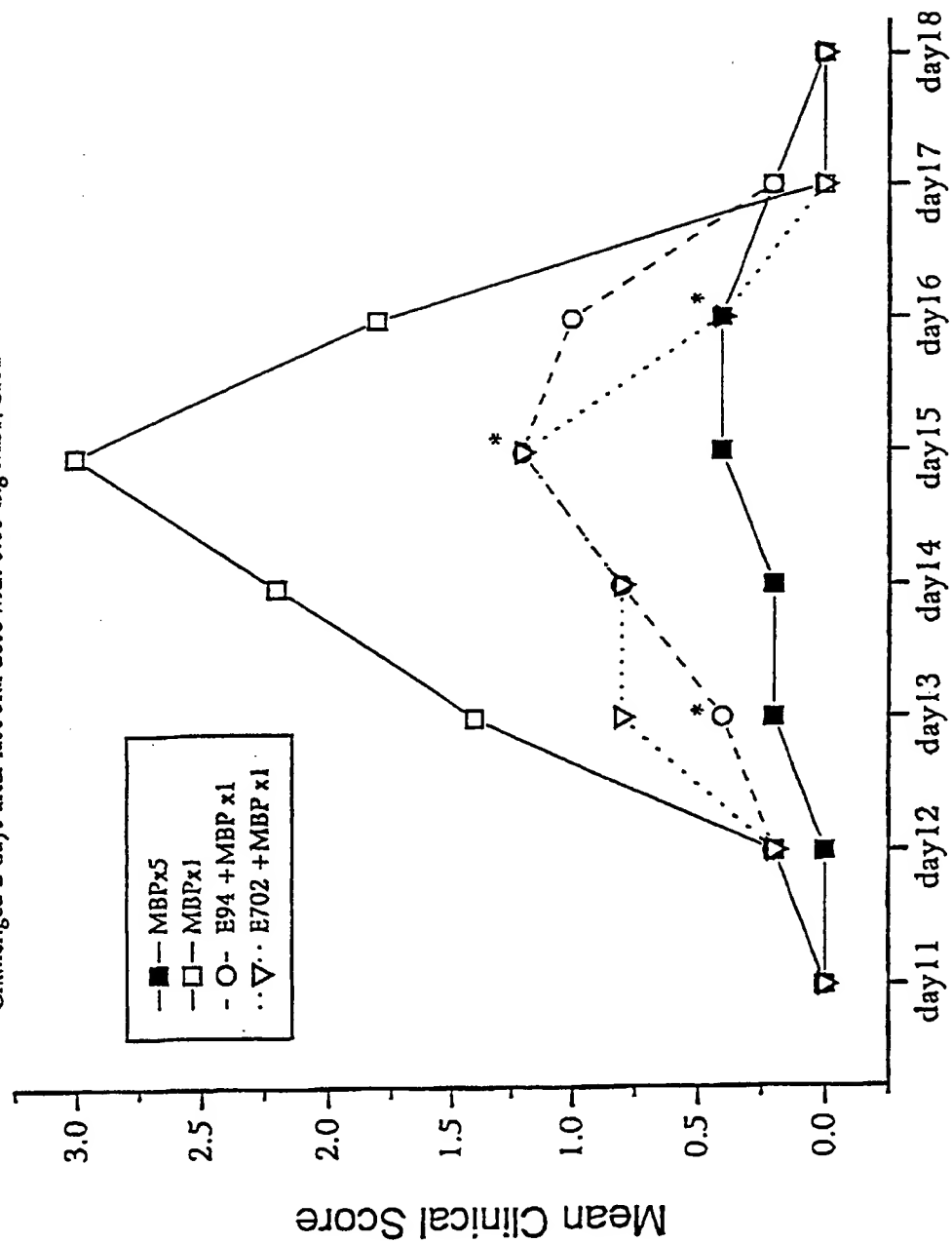
FIG. 9

Single oral dose 1.0 mg Ovalbumin +/- Delivery agent
Subcutaneous challenge (0.1 mg Ova/CFA) week 3
DTH week 5.



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FIG. 10
Female Lewis rats dosed orally once or five times with MBP (1.0 mg/dose)
or once with MBP with delivery agents (300 mg/kg).
Challenged 2 days after last oral dose with 0.05 mg MBP/CFA.

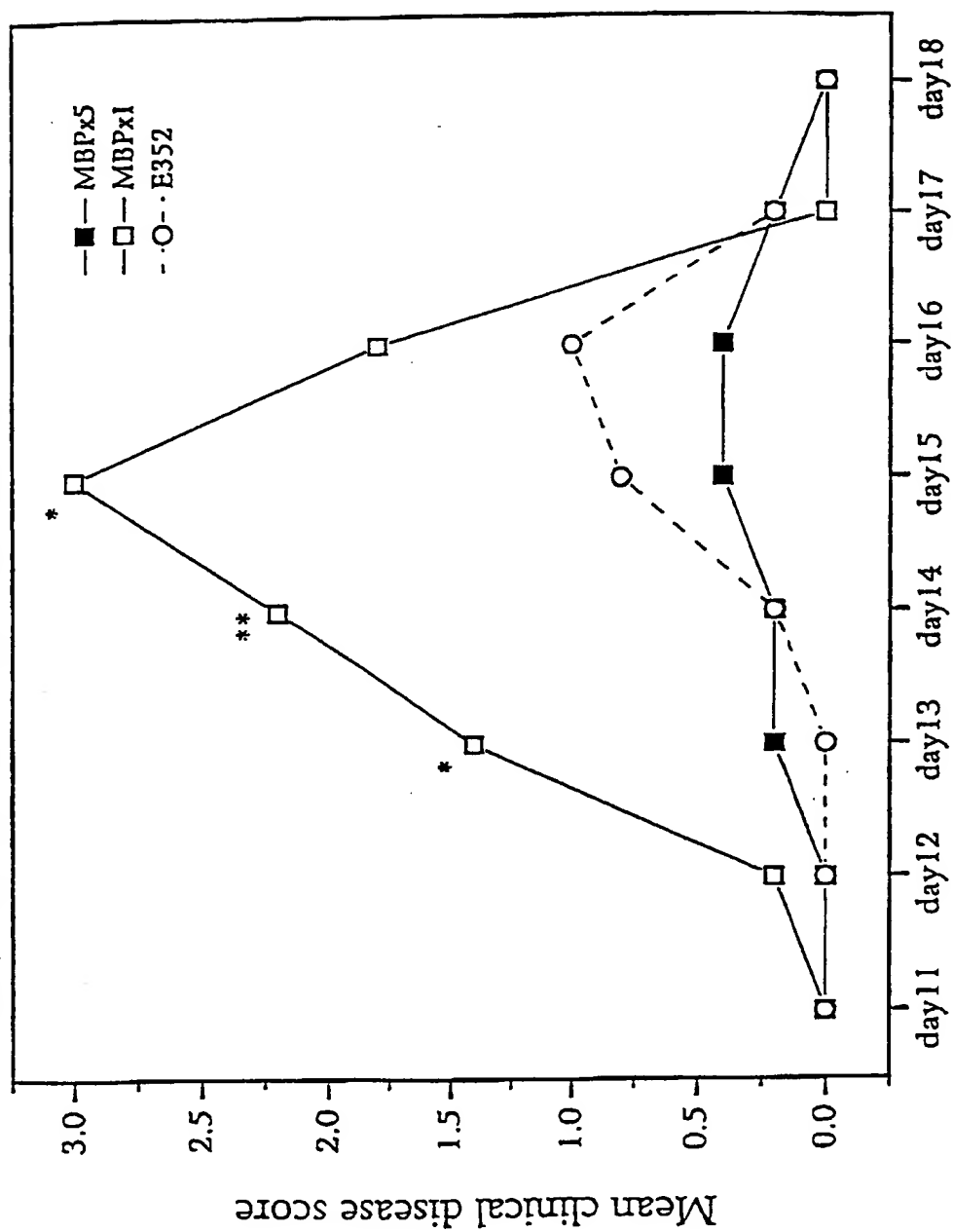


* $p < 0.05$ vs. MBP x 1

11/12

FIG. 11

Female Lewis rats dosed orally once or five times with MBP (1.0 mg/dose) or once with MBP with E352 (300 mg/kg).
Challenged 2 days after last oral dose with 0.05 mg MBP/CEA.



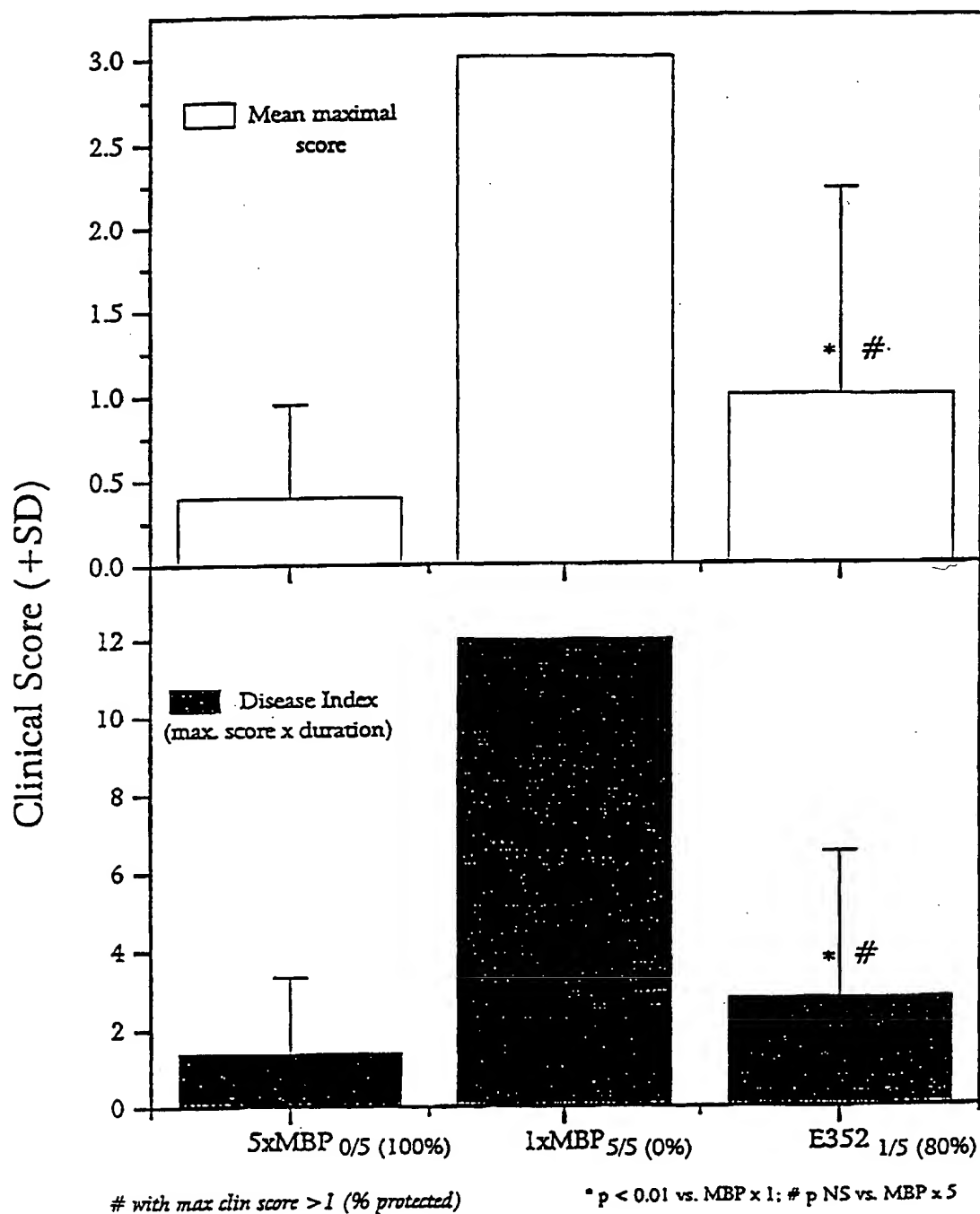
* $p < 0.05$ vs. E352; ** $p < 0.01$ vs. E352
MBP x 5: p NS vs. E352 at all time points

BME-2

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FIG. 12

Female Lewis rats dosed orally once or five times with MBP (1.0 mg/dose)
or once with MBP with E352 (300 mg/kg).
Challenged 2 days after last oral dose with 0.05 mg MBP/CFA.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/14676

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : A01N 37/18, 37/02; A61K 31/165 US CL : 514/617 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/617 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) REGISTRY, HCAPLUS search terms: deliver?(s)antigen#, structure search		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database on HCAPLUS on STN, Emisphere Technologies, Inc., Document No. 125:96071, Leone-Bay et al., "Modified amino acids as absorption enhancers for delivering active agents", abstract, WO 9612437 A1, 02 May 1996.	1-18
X	Database on HCAPLUS on STN, Cancer Research Campaign Technology Ltd., UK, Document No. 111:146800, Bagshawe et al., "Targeted delivery of antineoplastic prodrugs using an antibody-enzyme conjugate", abstract WO 8807378 A1, 06 October 1988.	1
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* *A* *E* *L* *O* *P*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	*T* *X* *Y* *A* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
Date of the actual completion of the international search 24 OCTOBER 1997		Date of mailing of the international search report 12 NOV 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer DWAYNE C. JONES Telephone No. (703) 308-1235